Angiogenesis and angiopoietins in human gliomas

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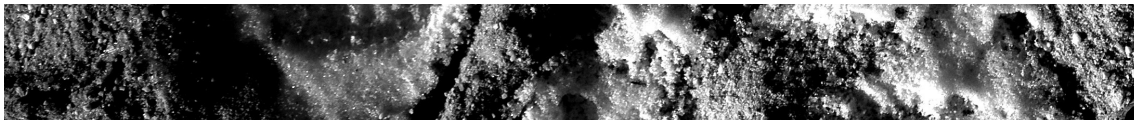
1. General introduction, aim and outline of the thesis

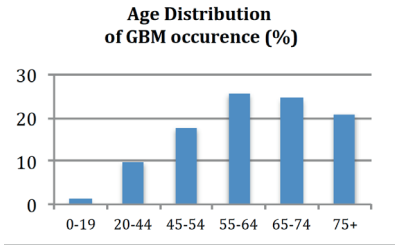


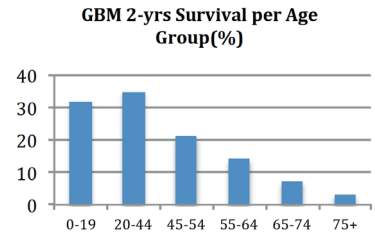
Introduction

Every year more than 500 patients in the Netherlands are diagnosed with a grade IV glioma (glioblastoma (GBM)), the most aggressive tumor that is intrinsic to the brain [28]. The prognosis of these patients is dismal and has remained so despite significant progress in the development of modalities that are available for treatment, whether it is surgery, chemotherapy, or radiotherapy. It is the need for improvement of current treatment that inspires researchers worldwide to search for alternative targets for therapy. The ability of GBM (and other tumors) to form new blood vessels, a process called angiogenesis, is such an alternative target. The transition of a tumor towards a state in which new tumor blood vessels are formed is known as the angiogenic switch. The angiogenic switch is under control of the Angiopoietin-Tie system. The role and relevance of the Angiopoietin-Tie system to glioma growth and development is the subject of this thesis. In this first chapter, an introduction into glioma biology and angiogenesis is presented, as well as the aim and outline of the thesis.

BOX 1 Gliomas

Gliomas are the most frequent primary tumors of the central nervous system parenchyma. The parenchyma of the central nervous system contains two main cell populations: neurons and glia cells. The glia cells have various functions such as the support of neuronal function and metabolism, the upholding of the blood brain barrier (BBB) and immune surveillance. Three types of glia cells are distinguished, i.c., Astrocytes, Oligodendrocytes, and Ependymal cells. Gliomas are neoplastic lesions that originate from these glia cells. Gliomas can be divided according to their cell of origin: astrocytomas, oligodendrogliomas and ependymomas. They are graded I-IV using the WHO classification and grading system [16]. In this grading system, grade I tumors are relatively 'benign', whereas grade IV tumors are 'malignant' . The terms benign and malignant are deliberately put in quotation marks here, because gliomas can not be labeled with these terms in their classical sense. That is, although the 'malignant' grade IV gliomas show infiltrative growth, they hardly ever form metastases in distant organ systems as do other malignant tumors. On the other hand, the more 'benign' grade II gliomas also show infiltrative growth, precluding a definitive cure with current treatment protocols. The grade I glioma is represented by the pilocytic astrocytoma and should be considered a separate entity outside the scope of this thesis. Astrocytomas and oligodendrogliomas form a spectrum of tumors that show increasingly 'malignant' features from grade II to IV and of which the lower grades, in time, develop into the highest grade, grade IV, also known as glioblastoma (GBM). The different grades can be distinguished by histological examination and represent different prognostic groups. The prognosis of patients with a GBM is very limited, with a 2yr survival rate of approximately 27% [26] when treated with a combination of chemo- and radiotherapy. In everyday practice, prognosis is probably worse [3], because this figure represents a selected trial population.





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Tumor Angiogenesis

In his 1971 paper [8] in the New England Journal of Medicine, Dr. Judah Folkman proposed that the ability of tumors to produce their own vasculature allows them to grow beyond a volume of roughly 3 mm^3. His hypothesis marked the beginning of a new field within the area of oncological research dealing with tumor angiogenesis. Since then, this field has grown tremendously and studies of the mechanism and relevance of angiogenesis in practically all tumor types have been performed. Folkman's hypothesis, ofcourse, holds promise for the possibility of targeting the vasculature in an attempt to revert tumors to their state of dormancy (<3 mm^3). Initially, hopes were high because the vasculature was considered an 'easy' target compared to the genetically instable and heterogeneous tumor cells. Not unexpectedly, things turned out to be more complex.

Early on, the growth factor Vascular Permeability Factor [24], later termed Vascular Endothelial Growth Factor (VEGF), was identified by Dr. Harold F Dvorak and his team and turned out to be one of the major players in angiogenesis. VEGF is produced under various fysiological and pathological circumstances and can be produced by a range of cell types such as tumor cells. VEGF actually designates a family of related growth factors sharing three transmembrane tyrosine kinase VEGF receptors (VEGFR1-3). The family consists of VEGF-A, -B, -C, -D, and Placental Growth Factor (PlGF), of which VEGF-A is the most well known and probably most potent [5]. Of VEGF-A, multiple isoforms are known that are formed by alternative splicing of the mRNA [19]. The various members of the VEGF family and the isoforms have variable affinities for the three VEGFRs. There is a complex mechanism of agonism and competitive antagonism between the VEGF family members, as well as a complex interplay between the receptors, including dimerization of receptors and the occurence of receptors as decoys. On the whole, VEGF contributes to tumor angiogenesis by stimulating survival, proliferation, and migration of endothelial cells. In addition, similar effects on tumor cells have been suggested [15].

Approximately two decades later, the driving force behind VEGF was identified: Hypoxia Inducible Factor-1 (HIF-1) [10, 23]. HIF-1 is a nuclear heterodimeric complex of HIF-1α and HIF-1β that can bind so-called hypoxia response elements (HREs) of genes to promote their expression. Under normal (i.e., normoxic) circumstances, HIF-1α, located in the cytoplasm of cells, is rapidly degraded by the proteasome. During hypoxia, HIF-1α is stabilized, can translocate to the nucleus, and, together with HIF-1β, can bind HREs of more than 800 target genes [22]. Through its target genes HIF-1 can promote not only angiogenesis, but also stem cell maintenance, metabolic reprogramming, autocrine growth factor signaling, epithelial-to-mesenchymal transition, invasion, metastasis, and resistance to therapy [22].
In many tumors HIF-1 activity is increased. This may be induced by intratumoral hypoxia, but can also be the result of loss of tumor suppressor genes or gain of oncogenes. The most illustrative example of the latter is the haemangioblastoma of patients with von Hippel-Lindau disease. In these patients, a mutation in the vHL-gene renders the protein dysfunctional. The direct consequence of this is an increased stability of HIF-1α despite the absence of hypoxia. In gliomas, there is circumstancial evidence for both mechanisms (hypoxia driven and gene mutation driven HIF-1 expression). In human glioma cell lines, there is a high expression of HIF-1 also under normoxic conditions [14]. On the other hand, the expression pattern of HIF-1 in glioblastomas (predominantly in the perinecrotic areas) suggests an oxygen concentration dependent mechanism, especially when compared to the diffuse expression in hemangioblastomas [30].

The VEGF gene is one of the target genes of HIF-1, as are other genes that are involved in angiogenesis, such as stromal derived factor (SDF), placental growth factor, platelet derived growth factor B (PDGF-B), and Angiopoietin-1 and -2 [22]. Increased activity of HIF and subsequent upregulation of VEGF sets off a sequence of events that, together, forms the process of angiogenesis and leads to the formation of new vessels. These events and the process of tumor angiogenesis as a whole are generally limited to the microvasculature, consisting of arterioles, capillaries and postcapillary venules.

In a schematic description of the process of tumor angiogenesis, a chronological order is ascribed to the events and the process is viewed as presented in figure 1. In tumors that are clinically relevant, in all likelihood, the various events that form the process of angiogenesis are all present, to a greater or lesser extent, at the same time.

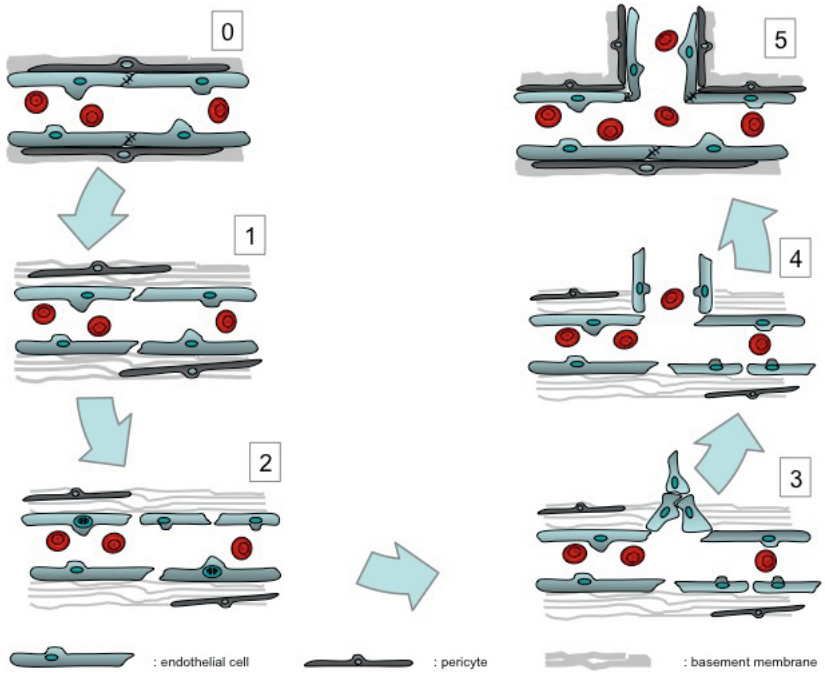

Figure 1: Schematic representation of the events that constitute the process of sprouting angiogenesis. The normal vasculature [0] has to be broken down for the endothelial cells to be able to proliferate and migrate. This vascular desintegration [1] is characterized by break down of the basement membrane and loosening of the pericytes. In doing so the endothelial cells are liberated and they start to proliferate [2] and migrate [3]. The sprout of endothelial cells that has formed subsequently gains vascular functionality by forming a tube through which blood can be transported (tube formation [4]). In the last step, the neovessel is again covered by pericytes and basement membrane in a process called maturation [5]), making the endothelial cells less dependent on external survival factors.

During pathological tumor angiogenesis, these events may very well occur to a variable extent. Some of the steps may be incomplete, leaving part of the vasculature dysfunctional or fragile. This is especially apparent for the process of maturation, which can vary extensively between tumor types and between patients [29] (see also chapter 3 of this thesis).

BOX 2 Various types of tumor neovascularization and vascular remodelling

Tumors can employ various strategies for ensuring an adequate blood supply. In the literature, the term tumor "angiogenesis" in its broadest sense may therefore include other mechanisms than the process described here, such as vessel cooption, intussusception, and vascular mimicry. Still other mechanisms, which are outside the scope of angiogenesis, such as vasculogenesis, may be involved. The process described here can, more specifically, be called "classical sprouting angiogenesis", and is the most extensively studied mechanism. For practical purposes, here, the more general term "angiogenesis" will be used to refer to classical sprouting angiogenesis unless stated otherwise.

The initial proposition by Dr. Judah Folkman, claiming that angiogenesis is necessary for tumors to grow beyond 3 mm^3, implies the possibility of the existence of pre-angiogenic or so-called dormant tumors. The concept and reality of these dormant tumors has by now been generally accepted [1]. The transition of dormant tumors towards angiogenically active neoplasms is known as the angiogenic switch. The Angiopoietin-Tie system is central to this switch because it controls the first step of tumor angiogenesis: vascular desintegration.

The Angiopoietin-Tie system

The Angiopoietin–Tie (Ang-Tie) system consists of the transmembrane tyrosine kinase receptors Tie-1 and Tie-2 ("Tie" stands for "**T**yrosine kinase with **I**mmunoglobulin and **E**GF homology domains") as well as the ligands Ang-1, -2, and -4 (Ang-3 is a murine ortholog of Ang-4). All Angiopoietins bind Tie-2, whereas Tie-1 is an orphan receptor (to this day no ligand for this receptor has been identified).

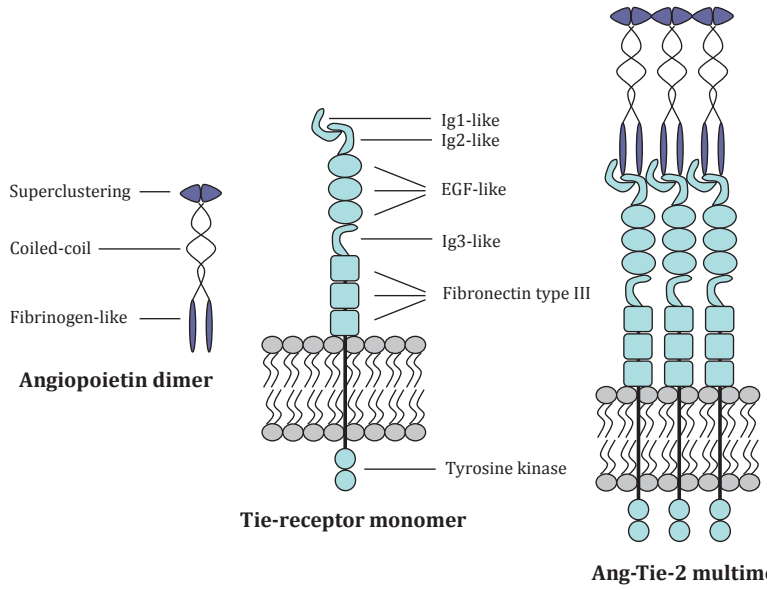

Figure 2: Schematic representation of the Angiopoietin molecules, the Tie-2 receptors, and their multimerization upon ligation (adapted from [4, 13]).

Embryological studies have shown that the Ang-Tie system is involved in vascular morphogenesis. Although the development of the primary capillary plexus seems independent of this system, further development of the vascular system through sprouting angiogenesis fails in Tie-2 or Ang-1 deficient mice and leads to early embryonic death [20, 27]. These mice also have insufficient haematopoiesis and deficient development of the endocardium. Tie-1 deficient mice die later during development due to widespread edema as a consequence of loss of vascular integrity. Ang-2 deficiency in mice does not lead to embryonic lethality [9]. However, these mice do show defective vascular remodeling . For instance, after birth, the physiological regression of the hyaloid vessels of the embryonic lens does not occur. These and other findings have led to the general concept that Ang-1 signals through Tie-2 and that Ang-2 functions as antagonist of Ang-1 [17]. The two Angiopoietins are thought to work as a balance, were dominance of Ang-1 is responsible for guarding vascular quiescence, whereas dominance of Ang-2 is necessary for vascular remodeling. Although Tie-1 and Tie-2 were initially thought to be endothelial cell specific, it is now known that they are also expressed by haematopoietic cells and tumor cells (see also chapter 5). While Tie-2 is mostly constitutively expressed by endothelial cells, Tie-1 expression seems highly regulated. Ang-1 is expressed by vascular mural cells as well as by some non-vascular cells (e.g., fibroblasts and tumor cells). After secretion, it can adhere to the extracellular matrix. Ang-1 is constitutively expressed, but can be upregulated, for instance during angiogenesis. Ang-2 is mostly produced by endothelial cells. It is stored in the Weibel-Palade bodies and can be released quickly to facilitate rapid endothelial cell responses [7]. This potential for rapid response relates to the important additional role of the Ang-Tie system in inflammation, where it is involved in the adhesion and extravasation of leukocytes [6].

Both Ang-1 and Ang-2 are clustered in multimers [4]. Ligation of Tie-2 leads to multimerization of the receptor. Because conformational changes of the Tie-2 receptor after ligation are minimal, it seems likely that the clustering of receptors is primarily responsible for phosphorylation. The complete process of binding, clustering and phosphorylation has not been fully elucidated and it is not exactly known what the basis is for the different effects of Ang-1 vs. Ang-2. Either a difference in multimeric states or a difference in 3D conformality of ligand binding may lead to variable arrangements of the Tie-2 clusters with variable biological effects [4]. The effects of Tie-2 activation by Ang-1 have been shown to be context dependent. In confluent endothelial cell monolayers, the Tie-2 multimers are translocated to the cell membrane that faces a neighbouring endothelial cell and form so-called trans-associations. In other words, the transmembrane Tie-2 multimers of two neighbouring cells are connected by Angiopoietin multimers. In this situation, survival and vascular quiescence are promoted. On the other hand, when Angiopoietin-1 ligates Tie-2 on an endothelial cell in isolation, then multimerization of Tie-2 occurs, but the complex binds angiopoetin-1 multimeres that are sequestered in the extracellular matrix. Tie-2 phosphorylation, in this case, promotes migration and proliferation. This context dependency possibly explains reports of paradoxal, opposing effects of Ang-1 on angiogenesis. It seems likely that in quiescent endothelial cells Ang-1 promotes and protects the quiescent state. However, once angiogenesis is up and running, Ang-1 may very well support this process.

Because a shift in the angiopoietin balance towards Ang-2 appears necessary to initiate vascular remodeling, and because Ang-1 is constitutively expressed, much interest has gone out to the

mechanism of Ang-2 upregulation. As stated, Ang-2 can be released from the endothelial cells quickly from the Weibel-Palade bodies. During tumor angiogenesis , however, there is also an increase in Ang-2 gene expression, and, possibly, a change in post transcriptional regulation. Upregulation of the Ang-2 gene in endothelial cells can be induced by HIF , TNFa, high glucose levels [25], VEGF, FGF-2, and PMA [11]. Simon et al [25] identified a HRE in the promotor region of the Ang-2 gene.

Besides its role in the regulation of vascular quiescence/remodeling and inflammation, the Ang-Tie system is involved in non-vascular functions. As stated above, the Ang-Tie system is instrumental for embryonic haematopoiesis, but also maintains adult haematopoiesis by contributing to the upholding of the haematopoietic stem cell niche. Ang-1 also has neuroprotective effects. Some of these effects may be independent of Tie-2 and could well be mediated by the binding to integrins [21].

Angiogenesis in Gliomas

Gliomas are infiltrative tumors and individual tumor cells may be encountered in the brain parenchyma at a distance of cm's from solid tumor mass. This infiltrative growth and the migration of glioma cells shows preference for the perivascular space. This kind of recruitment of the existing vasculature by the tumor cells is called cooption [18]. Cooption garantuees an adequate supply of oxygen to the migrating and proliferating tumor cells and seemingly obviates the need for an angiogenic response. Nonetheless, in GBM's, such a response does occur. In fact, together with necrosis, the occurence of microvascular proliferation forms the main charateristic distinguishing GBM's from their lower grade counterparts [16]. In the majority of these patients a switch towards this angiogenic response can not be clinically recognized. Patients most frequently present with a so-called primary glioblastoma (grade 4 glioma that has not evolved from a low-grade glioma). These lesions have a high angiogenic potential long before they are clinically detected. Less frequently, patients first present with a low grade glioma after which the lesion progresses towards a high grade lesion during clinical follow-up. One may argue that an angiogenic switch can be observed in such a case because there is a clear increase in vascular abnormalities and a clear difference in for instance microvascular density between the grades (chapter 2). However, to liken the progression of a low grade glioma to the angiogenic switch would be a gross oversimplification. For one, the radiological characteristic of high grade gliomas that we associate with the angiogenic response (i.e. contrast enhancement) may be present in a considerable part of low grade gliomas [2].

Preclinically, however, the transition of a preangiogenic lesion towards a tumor with actively proliferating endothelium has been well studied in a glioma tumor model [12]. In this model, the following mechanism of the initiation of the angiogenic response has been described (figure 3): The preangiogenic glioma lesion, coopting the existing cerebral vasculature, induces an upregulation of angiopoietin-2 in the endothelial cells that triggers the initial vascular desintegration. In this state, the endothelial cells are susceptible to apoptosis promoting factors. Because of this, vascular degradation occurs leading to local hypoxia. The subsequent upregulation of VEGF provides the remaining endothelial cells with the necessary survival signalling and induces proliferation, thereby starting up the process of tumor angiogenesis.

Whether this model holds true for the initial outgrowth of (all) human glioblastomas is uncertain. As stated earlier, a high level of HIF expression may be present in glioma cells regardless of the hypoxic state of the environment [14], allowing for the possibility of an angiogenic response in the absence of hypoxia. However, the model has two important assets: 1. It is consistent with the clinical, radiological, and histopathological appearance of human glioblastomas (i.e. an infiltrating, vessel cooptive tumor with a hypoxic, necrotic core and a vital, angiogenically active rim). 2. Regardless of the role of hypoxia, the model does emphasize the importance of the Ang-Tie system for initiation of the angiogenic response in gliomas. Additional preclinical glioma research has supported this claim. However, the role and relevance of the Ang-Tie system in the clinical setting has not been fully elucidated.

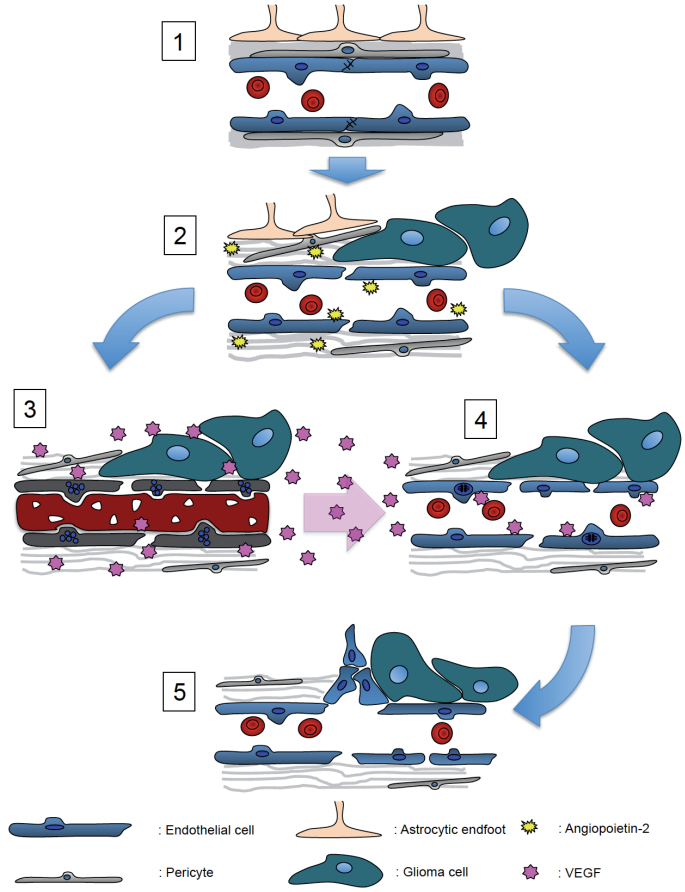

Figure 3: The normal cerebral vascular (1) shows a high level of integration of endothelial cells, pericytes , basement membrane and astrocytic endfeet. Perivascular growth of tumor cells (2) induces release and upregulation of Ang-2 in the endothelial cells and a subsequent desintegration of the vascular constituents. Locally, due to increased endothelial cell vulnerablity and subsequent apoptosis, the microvessels become occluded and the environment becomes hypoxic (3). As a consequence, VEGF production increases. In adjacent vessels, VEGF acts as a survival factor for the endothelials cells and leads to endothelial cell proliferation (4). Migration of the proliferating endothelial cells is facilitated by the Ang-2 induced vascular desintegration (5).

Aim and outline

The role and relevance of the Ang-Tie system in human glioma growth and development is the main subjects of this thesis.

In **chapter 2**, the analysis of glioma vasculature is reviewed. The review provides insight into the various techniques that can be used to study the vasculature and it discusses the unique characteristics of the microvasculature of the brain and gliomas that are important to consider when applying these techniques. Also, it examines the relevance of the assessment of tumor vasculature for the diagnosis and treatment of high grade gliomas.

In **chapter 3**, the extent of angiogenic activity in high grade gliomas is compared to that of ependymomas. This chapter illustrates the intensity of the angiogenic response in high grade gliomas. Several charateristics of the glioma vasculature that are of relevance to the angiogenic process are assessed, among which the Ang-Tie system.

The relevance of the expression of the Angiopoietins and the Tie-2 receptor and its relation to high grade glioma patient survival is the main subject of **chapter 4**.

Whereas chapter 4 deals with the expression of the angiopoietin-Tie2 system on a mRNA level, **chapter 5** describes these factors in high grade gliomas on a protein level. It discusses their expression pattern and studies the cell types that are involved, thereby introducing a different view on the role of the Ang-Tie system compared to the preceding chapters .

In **chapter 6** the changes in the vasculature in reaction to combined therapy (radiation and COX-2 inhibition) are studied in a preclinical model. The involvement of the Ang-Tie system in the response to therapy is analyzed.

In **chapter 7**, the findings described in the previous chapters are summarized and the relevance of these findings for current high grade glioma diagnosis and treatment is discussed. Also, possible future developments are anticipated.

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